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The decay of X-ray induced free radicals in plant seeds and starch

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With 6 figures in the text

ABSTRACT

The fate of the X-ray induced free radicals in living seeds and amylopectin of different water contents has been studied by means of electron spin resonance absorption. The same amount of free radicals is primarily produced in the seeds independently of their water content.

The radicals remain unchanged in the driest samples but an increasing proportion of them decays as the water content increases. The initial, relatively rapid decay which is apparently second order falls to a semiconstant level that decreases very slowly. The observed growth inhibitory effects at the different water contents are found to be proportional to these semiconstant levels. The radicals induced in amylopectin are very sensitive to oxygen. The reaction in air is first order with respect to the radical concentration; its velocity increases with the water content.

By means of electron spin resonance (esr) it was recently shown that irradiation by X-rays induced relatively long lived free radicals in living material—embryos cut from resting barley seeds [1]. This material was, however, not the best possible since the embryos were injured by the excision and had a limited life time. The present work deals with extended experiments on intact seeds, aiming at an elucidation of the mechanisms of action [2] of ionizing radiations. The studies center round the problem of how the water content of biological materials [3–8], resting seeds and also hydrophilic colloids [9, 10] protects against radiation damage. This protection is known to be most pronounced when there is 16 to 20 % H_2O, the sensitivity to irradiation being greater for lower as well as higher water contents.

It was thus of primary importance to be able to obtain comparable results in the esr spectrometer on samples of different water contents and, consequently, varying dielectric losses in the microwave field. This is an intricate problem since the sensitivity of the spectrometer is a quite complicated function of the dielectric properties, dimensions, and orientation in the resonant cavity of the sample and of its container. The cavity of the instrument used is a rectangular resonator operated in the TE_{012} mode [11]. A cylindrical sample can be centered in the cavity and is then also centered at the electrical node of the microwave field. The losses must hence increase and the sensitivity gradually decrease with increasing diameter of the sample. One way to correct for these losses is thus to measure each sample in tubes of different diameter, divide the signal amplitudes by the effective sample volume or weight and extrapolate the data to zero diameter.

In order to make it possible to measure at small tube diameters the seeds to be studied had to be small in size. The material must further have properties making it suitable for parallel biological, e.g. cytological, studies. The seeds of the grass, *Agrostis stolonifera* (variety Z 104 from Weibull's Plant Breeding Institute, Landskrona), were found to be ideal from these points of view. They are small enough (thousand grain weight 0.08 g) to be easily filled into a glass tube of 1 mm internal diameter; they are tetraploid [12], and hence rather insensitive to irradiation, which permits the free radicals and the biological effects to be measured on the same sample.

Irradiation was carried out as before [1, 13] with 175 kV X-rays filtered through 2 mm aluminium, a dose rate of 800 r min^{-1} being used. In order also to obtain signals of suitable height from the small amount of sample that could be placed in the narrow tubes (1 mm internal diameter), it was necessary to use rather high doses. The long irradiation times and the rapid decay of the radicals in seeds of higher water content, restricted the studies to seeds of water contents less than about 10 %. For comparison some experiments were performed to investigate the influence of water content and oxygen tension on the decay of the free radicals in irradiated amylopectin.

Experimental

Seeds of *Agrostis stolonifera* were kept for 2 weeks in air of 0, 20, and 30 % relative humidity [5]. After this time equilibrium had been reached and the three samples had a water content of 3.1, 8.5, and 9.9 %, respectively. Part of each sample was irradiated with X-rays for 200 min. (total dose 160 kr). As soon as possible after irradiation the seeds were measured in the esr spectrometer in tubes of three different internal diameters; 1, 2, and 3 mm, respectively. In the first period of time the spectra were recorded with the shortest possible intervals and they were followed for about 15 days. The unirradiated seeds were measured before and after. Between measurements the samples were stored at room temperature (20°C) in desiccators with the air adjusted to the relative humidities indicated. The temperature in the cavity of the esr spectrometer was not controlled but was found to be approximately 18°C. In each case the weight of the sample was determined and the effective weight located inside the microwave cavity was calculated. The amplitudes of the derivative curves recorded were divided by the corresponding effective weights in order to obtain comparable data.

In a similar way samples of amylopectin were equilibrated with air of 0 and 70 % relative humidity. The water contents thus obtained were 4.74 and 17.8 %, respectively. The samples were irradiated in vacuum. The sealed tubes were long enough to allow irradiation of the whole sample at one end of the tube, while the other end was shielded by lead. The measurements in the resonance spectrometer were made after transferring the samples to the unirradiated part of the tube [13]. The signals were followed for about 20 hrs with the sample in vacuum, after which time the tube was opened, the powdered sample was shaken to ensure good contact with the air and the measurements were continued.

Results

Fig. 1 shows a typical record of the derivative of the absorption of irradiated seeds and the corresponding blank for unirradiated material. A fraction of the blank signal is due to paramagnetic metal constituents of the glass. Since the signals have about

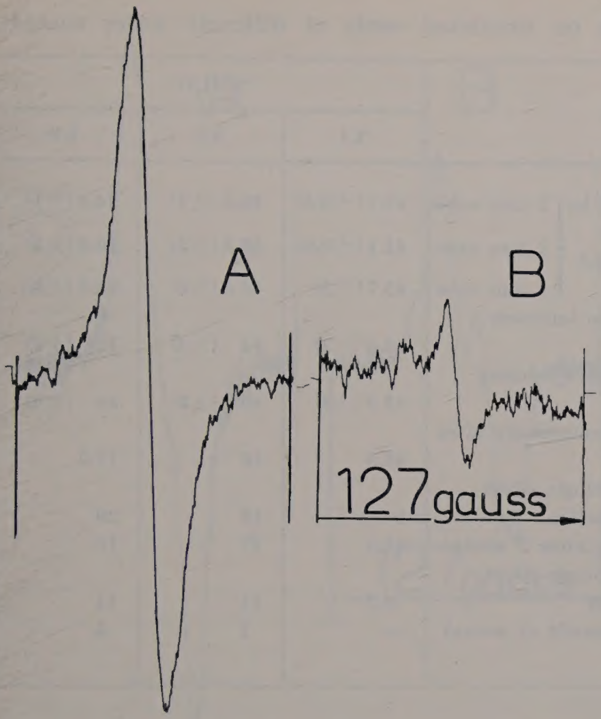

Fig. 1.

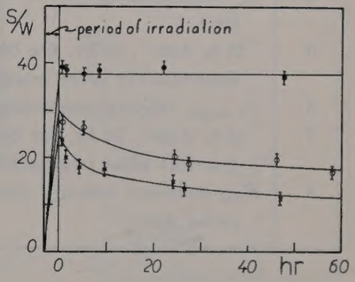

Fig. 2.

Fig. 1. Derivative records of esr absorption of seeds with 3.1 % H_2O: (A) after irradiation with 160 kr, gain 5/100. (B) before irradiation, gain 1/10. Conditions: Microwave frequency 9360 Mc/sec. Magnetic field: 127 gauss increasing field sweep, 12 min sweep time, 11 gauss peak to peak modulation at 70 c/sec. Time constant of phase sensitive detector was 40 sec.

Fig. 2. Time dependence of signal amplitudes from X-ray irradiated seeds of different water contents: ● 3.1 %, ⊙ 8.5 %, and × 9.9 % H_2O. The vertical lines indicate the standard deviations. *Abscissa:* time in hours. *Ordinate:* signal amplitude divided by the effective weight, *S/w*.

the same width, correction for the blank signal was made by mere subtraction of its peak to peak amplitude from that of the irradiated sample. The blank signals remained practically constant during the fourteen days over which the experiment extended.

The corrected signal amplidudes were divided by the effective weights of the corresponding samples and the data plotted *versus* time as is illustrated in Fig. 2 for the case of tubes of 3 mm internal diameter. It is seen that the signals from the driest sample did not change with time, while those from the samples containing more water decayed rapidly during the first day but much more slowly later on. The mean values of the signals from the dry seeds were taken for the different tube diameters and the standard deviations were calculated (Table 1). The standard deviations of the individual determinations are indicated in Fig. 2. The data for the wetter samples had to be extrapolated to the end of the irradiation period. This was most easily done with the data from the samples in the 3 mm tubes, but the operation was more difficult with decreasing tube diameters because of the increasing errors in the individual determinations. However, reliable data were obtained by making the extra-

Table 1. Summary of results on irradiated seeds of different water content.

Line No.	Line item		% H_2O		
			3.1	8.5	9.9
1	$S_{t=0}$ (signal ampl. divided by effective sample weight (S/w) extrapolated to end of irrad. period)	3 mm tube	37.7 (±0.5)	29.5 (±1)	24.5 (±1)
		2 mm tube	41.1 (±0.9)	38.5 (±2)	29.5 (±2)
		1 mm tube	43.7 (±3)	42.5 (±6)	28.5 (±6)
2	$S_{w=0}$ ($S_{t=0}$ extrapolated to infinitely small sample)		43.5 (±3)	44 (±4)	32 (±4)
3	S_{tot} ($S_{w=0}$ corrected for decay during irradiation)		43.5 (±3)	46 (±5)	38 (±6)
4	S_c (semi-constant level, immediately after irr.)		43.5	25	17.5
5	"25 % dose", in kr, for biologic effect immediately after irradiation		9	16	20
6	$S_{2\ \text{weeks}}$ (signals remaining after 2 weeks)		43.5	22	10
7	"25 % dose", in kr, for biologic effect 8 weeks after irradiation		3.5	11	14
8	K_{rel} (relative velocity constant of second order decay)		—	1	4

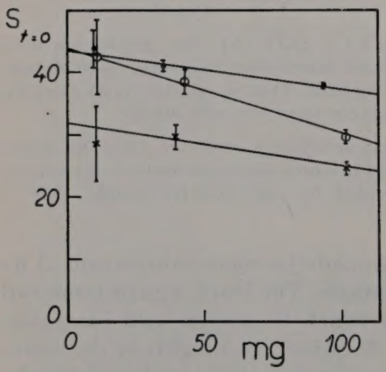

Fig. 3. Correction for dielectric losses by extrapolation to infinitely small sample. *Abscissa:* effective weight of sample. *Ordinate:* $S_{t=0}$, i.e., signal amplitude per effective weight extrapolated to end of irradiation period. The estimated standard deviations are indicated by vertical lines. ● 3.1 %. ○ 8.5 %, and × 9.9 % H_2O.

polation in relation to the levels of the signals for the different tube diameters in the later part of the experiment, 20 to 400 hrs after irradiation. Because of the errors involved in the procedure, the deviations of the extrapolated data have been set equal to double the standard deviations of the values for the dry samples, as is indicated in Table 1. In order to correct for the losses in the samples these extrapolated values are plotted *versus* the effective sample weight, Fig. 3, and extrapolations made to infinitely small samples. The data thus obtained (Table 1, line 2) are proportional to the amount of radiation produced free radicals present in the three samples at the end point of the irradiation period. In the wet samples these amounts are smaller

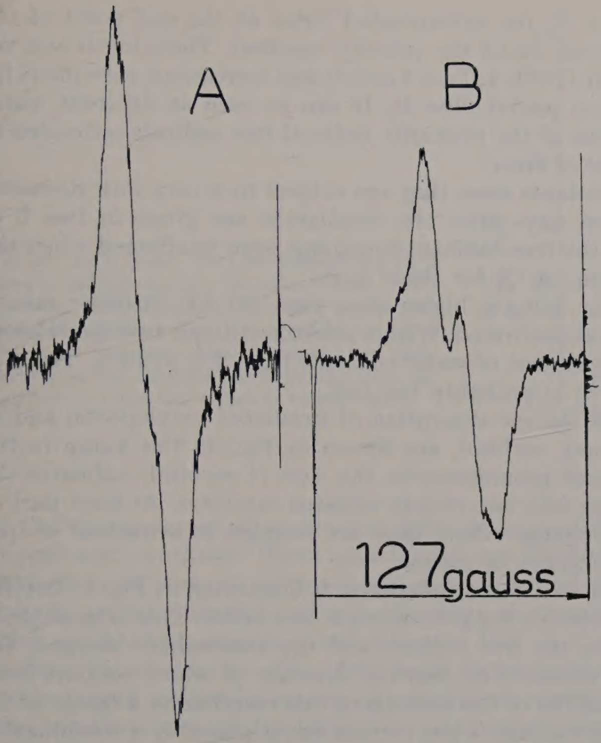

Fig. 4.

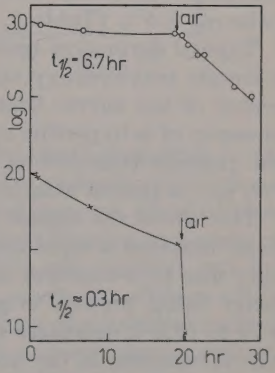

Fig. 5.

Fig. 4. Derivative records of esr absorption of (A) irradiated amylopectin. 4.74 % H_2O, gain 5/100, and (B) irradiated crystalline sorbitol, gain 2/100. 8.5 gauss peak to peak modulation at 70 c/sec. Time constant of detector 10 sec. Other conditions as stated in Fig. 1.

Fig. 5. The decay of irradiation-induced free radicals in amylopectin of water content 4.74 % (⊙) and 17.8 % (×). *Abscissa:* time in hours. *Ordinate:* logarithm of derivative signal amplitude.

than the amounts of primarily produced free radicals because of decay during the period of irradiation.

In order to be able to correct for this decay it is necessary to know the reaction mechanism involved or at least to formulate a law that approximately fits the data for the period immediately after irradiation. Several combinations of zero, first, and second order reactions were tried, but the experimental data were best fitted by the simple assumption that part of the radicals remained at a constant level while the rest decayed by a second order reaction, i.e., the decay fitted the differential equation

$$\frac{dS}{dt} = -k\,(S - S_c)^2$$

and its integrated form
$$\frac{1}{S_0 - S_c} - \frac{1}{S - S_c} = k\,t,$$

where S is the signal at time t, S_0 the extrapolated value at the end point of the irradiation, S_c the constant level, and k the velocity constant. These levels and velocity constants were calculated (Table 1, lines 4 and 8) and corrections were made for the decay during the irradiation period (line 3). It can be seen at different water contents, that the concentrations of the primarily induced free radicals estimated in this way agree within the limits of error.

The S_c levels are not real constants since they are subject to a very slow decrease. The signals remaining fourteen days after the irradiation are given in line 6 of Table 1. These signals due to the free radicals remaining were unaffected when the seeds were treated with 60 atm. of O_2 for three days.

In a more recent experiment using a higher dose rate (50 kV, 20000 r min^{-1}) the initial second order decay was confirmed. Within a factor of about two the velocity constant was found to be independent of water contents from 4 % to 16 %. The K_{rel} value at 8.5 % (Table 1, line 8) is probably too low.

Typical derivative records of the esr absorption of irradiated amylopectin and in a simple polyhydroxy compound, sorbitol, are shown in Fig. 4. The hump in the center of the curve, that is most pronounced in the case of sorbitol, indicates the presence of a hyperfine splitting into two closely situated satellites. At least part of the radicals thus belong to groupings where they are coupled to a nucleus of spin 1/2, i.e., a proton attached to oxygen or carbon.

The fate of the signals from irradiated amylopectin is illustrated in Fig. 5. Letting in air initiated a rapid decay, which a log plot shows is first order. This is most probably due to a reaction between the free radicals and the atmospheric oxygen, the latter being present in great excess. The fourfold increase of water content from 4.74 to 17.8 % decreases the half-life of the radicals in this reaction by a factor of 20. The mechanism of the decay in vacuum is less certain but is probably a combination of a second order recombination of the radicals, as in the case of the seeds, and a reaction with traces of oxygen left after the evacuation.

The radicals induced in crystalline sorbitol were not sensitive to atmospheric oxygen. They decayed at a very slow rate in the same way as has been previously observed for crystalline glycine [13, 1].

Discussion

When considering the biological significance of the measurements it must be remembered that the plant seeds constitute, chemically, a very complex system [cf. 14]. Besides the chiefly proteinous embryo, the seed contains an endosperm, the dominating constituent of which is starch, and seed coats, which are built up from cellulose and related polysaccharides. In addition low-molecular organic and inorganic compounds occur, chiefly in the embryo. Part of the esr absorption already exhibited by unirradiated seeds may be due to metal ions with an odd number of electrons, and/or to enzyme activities [15, 16, 17]. The difficulties in interpreting the radiation effects are increased by the fact that this "spontaneous" esr absorption in the controls is not certainly unaffected by the physiological state; thus, for example, it is almost doubled when the seeds are killed by heat treatment.

Enough data seem now to have been accumulated to permit the assumption that the primary changes leading to lesions of different kinds (mutation; chromosome aberrations; growth inhibition; death) occur in the nucleoproteins or nucleic acids

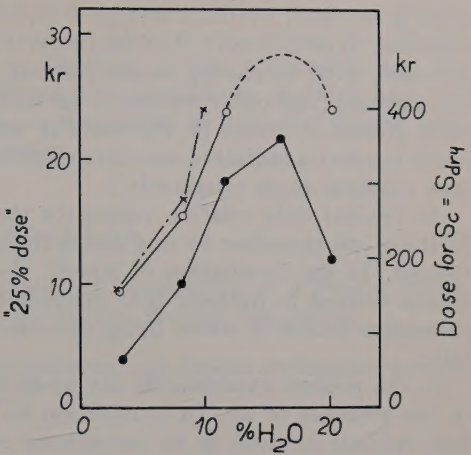

Fig. 6. "25 % dose", i.e., the dose giving 25 % growth inhibition, as a function of the water content, immediately (○) and 8 weeks (●) after irradiation. The doses needed to give the same amount of semiconstant free radicals as in the driest seeds have also been indicated (×) and the ordinate to the right.

of the chromosomes of embryonal cells, or in proteins (enzymes) partaking in the nucleic acid synthesis. When intact seeds are irradiated, free radicals are no doubt induced in all parts of the seeds and in a variety of chemical systems. Most of these radicals are not harmful and will disappear or remain without causing any damage. Some might represent chemical damages that in themselves are harmful and will produce visible lesions when the seeds are permitted to germinate. Others might produce such lesions by means of subsequent reactions. Since the harmful radicals only constitute a small fraction of the total signal measured, any correlation with the biological effects of the concentration and decay of the free radicals should be undertaken carefully. Similar care is necessary when considering the water content of the material: An average water content is measured, but the exact water content in the immediate vicinity of those molecules, the change of which leads to lesions, is unknown. With regard to the water content, however, the seeds are systems in equilibrium, and the water contents of different hydrophilic systems show very much the same dependence on the humidity of the air [cf. 18]. Since the radiation sensitivity of different, living or dead systems also exhibit about the same dependence on their water content [6, 10], a correlation of esr measurements to biological effects is permitted, at least in general terms and with due consideration to the uncertainties mentioned.

In the seed material used, the doses producing 25 % reduction of seedling height (at a control height of 20 mm) were determined (Fig. 6). In these experiments the seeds were sown on moist filter paper, at 20°C, a few hours after irradiation. It is seen that within the range of water contents studied with respect to the induction of free radicals, the radiation sensitivity varies by a factor of two or somewhat more, as 19 kr applied on seeds with 9.9 % H_2O produce the same amount of damage as 9 kr applied with 3.1 % H_2O. Within the limits of experimental error a given X-ray dose was found to induce the same number of free radicals at all the water contents studied, and the protective action of water, if operative on the free radicals, must therefore be a consequence of the later fate of the radicals.

In the present experiments the biological effectiveness of the X-rays seems to be proportional to the semi-constant plateau of free radical concentration reached within a few hours after irradiation (Figs. 6 and Table 1, lines 4 and 5). It could therefore be assumed, hypothetically, that the protective action of water, which below 16–20 % H_2O increases with increasing water content, is due to an increasing recombination of free radicals (2nd order reaction!). (A similar explanation, in more general terms, has been presented earlier [6, 9], where it was founded on the fact that with increasing water content a radiation-sensitive small molecule, the iodide ion, adsorbed on starch, was oxidized more effectively.)

At present only relative values for the free radical concentrations can be given. Nothing can therefore be said about the amount of energy consumed per free radical formed. In the irradiation of starch, the number of breaks in the polysaccharide chains seemed to indicate that excitations as well as ionizations were at work, the protective action of water being directed against the chemical effects of the former [10].

In the present experiments the seeds were irradiated and stored after irradiation in the presence of air. In comparison to the rapid decay, in the presence of air, of free radicals induced in an amorphous colloid such as amylopectin, the extremely slow decay of radicals once the "semi-constant" plateau is reached is very conspicuous. In this respect the seeds behave more like a crystalline compound (glycine [13], sorbitol, or glucose), a glass (e.g. quartz, Pyrex glass) or a plastic (e.g. teflon). Several data demonstrate that one important factor causing this later decay is due to reaction of the radicals with oxygen, as, for instance, the fate of the radicals upon the introduction of air in amylopectin irradiated in vacuo (Fig. 5). Gordy and co-workers [19] have also demonstrated a similar effect in irradiated proteins and nucleic acids.

A possible explanation of the high stability of free radicals in seeds as compared to starch, might be a low concentration of free oxygen in the former. This finding is in accordance with a low intracellular oxygen concentration expected from the fact that, whereas treatments by high pressures of oxygen induce high frequencies of chromosome aberrations in *Tradescantia* pollen [20] and in seeds [21] of barley, peas and horse-beans, the frequency of spontaneous aberrations is extremely low, especially in grasses. (A low intracellular oxygen tension would help to stabilize the genetic material.)

The esr measurements will probably give a basis for the interpretation of certain post-irradiation effects observed after irradiation of plant seeds. Caldecott *et al.* [22] allowed barley seeds, irradiated at a low and a high water content, to absorb water and germinate at different oxygen tensions. They found that a high oxygen tension doubled the radiation effect in the case of dry seeds, whereas those which had been irradiated at a higher water content were practically unaffected by the treatment. This can be understood if the action of oxygen, when it potentiates radiation injury, starts by its reaction with free radicals: These are present at a high concentration in the driest seeds, whereas most of them might have decayed before germination in the case where wetter seeds were irradiated.

It is possible that the increase of radiation injury occurring during storage of plant seeds [23, 24, 5] has to be interpreted on similar grounds. Although this after-effect develops more slowly in dry seeds, its total amount, as measured after 8 weeks' storage at 20°, is proportional to the amount of radicals at the semi-constant plateau (Fig. 6 and Table 1). This is valid in the range of low water contents treated in this study. At still higher water contents the after-effect increases again, possibly as a consequence of increasing metabolic activity and/or the greater mobility in the system which permits the radicals to take part in new types of reactions.

In summarizing it can be said that, because of the complexity of the living system studied, no definite conclusions can be drawn at present concerning the relation between the X-ray induced free radicals as observed by esr absorption and the biological effects on the same system. However, the correlations outlined here will probably prove fruitful as a working hypothesis that might be tested by refined techniques and by studies on simpler model systems.

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